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**RESEARCH NOTE**

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# Fermented Peel of *Citrus sunki* Hort. ex Tanaka Promotes Ethanol Metabolism and Suppresses Body Fat Accumulation

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**Abstract** *Citrus sunki* Hort. ex Tanaka is one of several Jeju-native citrus fruits. A number of biological properties for citrus fruits have been suggested, however little is known about those of *C. sunki*. The present study tested whether fermented product of *C. sunki* peel (FSP) might affect the activities of enzymes regulating ethanol metabolism. Effects on body weight gain as well as on fatty liver formation were also investigated. The activities of alcohol dehydrogenase and aldehyde dehydrogenase were stimulated remarkably by FSP. Excessive ethanol-induced cytotoxicity was also prevented by FSP in HepG2 cells. FSP decreased the weight gain and fatty liver formation induced by a high-fat diet in mice. From these results, FSP might be a potent source of neutraceuticals useful for preventing ethanol-induced health problems.

**Keywords:** fermented *Citrus sunki* peel, ethanol metabolism, weight gain, fatty liver

## Introduction

*Citrus sunki* Hort. ex Tanaka (*C. sunki*, Korean name, *jinkyool*) is one of 12 different Jeju-native citrus fruits exclusively cultivated in the Jeju region of Korea. Although 22 cultivars of Jeju native citrus have been documented since AD476 in ancient historical records (1), many of them have been replaced with other citrus fruits (mainly *Citrus unshiu*) because of their poor taste and lack of economic value. However, the economic significance of the diversity of biological species has recently been stressed. Therefore, studies to find novel functions from biological resources distributed in a restricted region are currently getting more interest with regard to neutraceutical functions.

Following a report analyzing the antioxidative activity of *C. unshiu* peels (2), changes in the flavonoid composition of Jeju-native citrus fruit peel during maturation has also been investigated (3). A recent study analyzed the antibacterial and anti-fungal activity of essential oil extracted from *C. unshiu* peel (4). Several classes of citrus phytochemicals, including monoterpenes (5), limonoids (6), and flavonoids (7, 8) have been recognized as effective chemopreventive agents in some animals. Other citrus components have been shown to be capable of suppressing inflammatory responses (9). Depression of blood pressure (2, 3) and prevention of dental caries (10) have also been shown for citrus flavonoids. Dried peel of *C. sunki*, known as *jinpee*, has been used as an essential component in most oriental prescriptions, however little is known about its biological functions or mechanisms of action. Our recent study showed the antioxidative- and anti-inflammatory activities of dried peel of *C. sunki* and its fermented product in different cellular systems (11). The fermentation of *C. sunki* peel helps to produce a number of beneficial metabolites as well as to develop edible products which are more easily digested (12).

The present study was performed to investigate the effects of fermented *C. sunki* peel (FSP) on ethanol metabolism and excessive ethanol-induced cytotoxicity, weight gain, and fatty liver formation.

## Materials and Methods

**Materials** *Citrus sunki* was obtained from different farms in the Jeju region of Korea and the peels were air-dried at room temperature. Powdered peel (100 g) was extracted with 1 L of 80% ethanol for 7 days at room temperature and passed through a Whatman GF/C filter. The ethanol fraction was removed from the supernatant with a rotating concentrator and the remaining water-fraction was freeze-dried (water extract of *C. sunki* peel, SP). Autoclaved SP mixture (5% sucrose, 50% SP in distilled water, w/w) was inoculated with 5%(w/v) *Lactobacillus plantarum* ATCC 8014 ($3\times10^6$ cells/mL) and 5%(w/v) *Saccharomyces cerevisiae* IFO 0203 ($4\times10^6$ cells/mL) and further incubated under anaerobic conditions for 7 days at 38°C. The fermented mixture (FSP) was freeze-dried, powdered, and stored at -20°C until used for experiments.

**Cells and animals** HepG2 (Korean Cell Line Bank, Seoul, Korea) cells were cultured in Dulbecco's Modification of Eagle's Medium (DMEM) with 10% fetal bovine serum in a humidified 5% $CO_2$ incubator. Male ICR mice aged 3 weeks (Daehan Biolink, Seoul, Korea) were fed with normal or high-fat rodent pellets (#101556; Dyets Inc., Bethlehem, PA, USA)

**Alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) assay** ADH and ALDH activities were measured as previously described (13) with minor

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modifications. The ADH fraction used for enzymatic analysis was obtained from S9 rat liver homogenate (Moltox Co., Boone, NC, USA), dissolved in 0.1% bovine serum albumin and aseptically filtered. The reaction mixture for ADH analysis consisted of 1.4 mL distilled water, 0.75 mL of 1 M Tris-HCl (pH 8.8), 0.3 mL of 20 mM $NAD^+$, 0.3 mL ethanol, 0.15 mL ADH fraction, and 0.3 mL test sample solution. After incubating at 30°C for 5 min, the absorbance at 340 nm was measured to verify the nicotinamide adenine dinucleotide (NADH) production rate.

The reaction mixture for ALDH assay consisted of 2.1 mL distilled water, 0.3 mL of 1 M Tris-HCl (pH 8.0), 0.1 mL of 20 mM $NAD^+$, 0.1 mL of 3 M KCl, 0.1 mL of 0.33 M 2-mercaptoethanol, 0.1 mL ADH fraction, and 0.1 mL test sample solution. The production rate of NADH from $NAD^+$ was determined from the absorbance at 340 nm after 5 min of incubation at 30°C.

**3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) activity** The measure of mitochondrial function was performed as described previously (14) with cells seeded on 24-well plates. HepG2 cells were pretreated with different concentrations of FSP for 30 min, and then further incubated with 5% ethanol for an additional 48 hr. Following treatment, the medium was removed from each well, and 200 mL of MTT reagent (Sigma, St. Louis, MO, USA) at a concentration of 1 mg/mL in RPMI-1640 medium without phenol red was added to each well. After 1 hr incubation at 37°C, the cells were lysed by the addition of 1 volume of 2-propanol and shaken for 20 min. Absorbance of converted dye was measured at a wavelength of 570 nm.

**Histological examination** Excised liver tissue was fixed with 10% buffered formalin and then embedded in paraffin. Tissue sections (10 μm) were stained with hematoxylin and eosin, and then photographed.

**Statistical analysis** Results are presented as the mean ± stardard deviation. Analysis of variance (ANOVA) was performed for each group. Duncan's test was also used to assess the significance of the difference for each treatment.

## Results and Discussion

**Effect of FSP on ADH and ALDH activities** Although citrus fruits have been reported to have antioxidant (15, 16), antitumor- (7, 8), and anti-inflammatory activities (9), little is known about their involvement in the regulation of ethanol metabolism. Ingested ethanol is metabolized into acetaldehyde by ADH in liver cells, and acetaldehyde is converted into acetate by ALDH which is ultimately excreted from the body as water and carbon dioxide. We tested whether FSP may enhance ADH/ALDH-mediated ethanol metabolism (Fig. 1A). Two different market-leading hangover drinks (CON and Y80) were used as positive controls. FSP stimulated ADH activity 2.3 fold over basal levels whereas CON and Y80 stimulated ADH activity by 1.9 and 2.1 fold, respectively. More significantly, stimulation of ALDH by FSP (1.8 fold) was also higher than those by CON and Y80 (1.2 and 1.4 fold, respectively). A previous study showed that Saeng-Maek-San, a medicinal herb complex, increased ADH activity thereby contributing to the protection of liver cell damage by alcohol (17), however it did not discuss the effect of Saeng-Maek-San on ALDH activity. It is widely recognized that acetaldehyde, which is more reactive and toxic than ethanol within the body, is the principle cause of alcoholic liver failure. Thus, the modification of acetaldehyde is a more essential step for diminishing alcoholic insults than the modification of ethanol itself. More effective stimulation of ALDH by FSP than the positive controls suggests the potential use of FSP in nutraceuticals to protect the liver from the alcoholic damage.

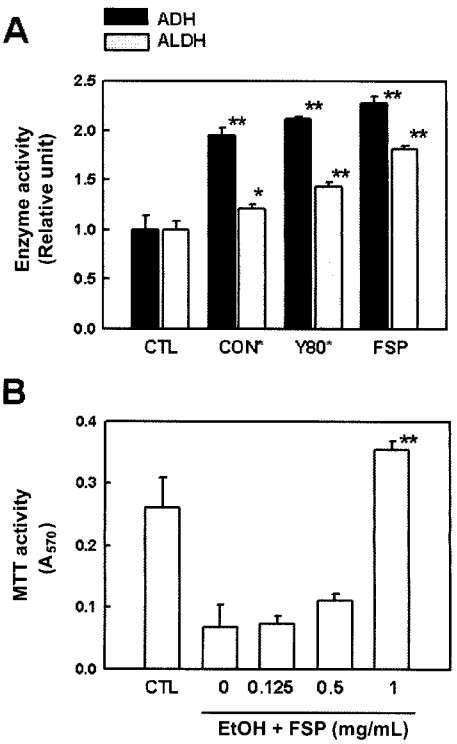


**Fig. 1. Effect of FSP on ADH and ALDH activities (A), and on ethanol-induced cytotoxicity in HepG2 cells (B).** CTL, control; CON*, hangover drink 1 (positive control 1); Y80*, hangover drink 2 (positive control 2); FSP, fermented *Citrus sunki* peel; EtOH, ethanol. *$p<0.05$, **$p<0.01$ compared to control.

**Protective effect of FSP against ethanol-induced cytotoxicity** Ethanol-induced liver damage in the human hepatoma cell line HepG2 has been reported and this experimental model represents the same pattern observed in human biopsies with alcohol-induced liver damage (18). Moreover, excessive ethanol results in the apoptotic cell death of HepG2 cells (19). The present study tested whether FSP may protect HepG2 cells from apoptotic death induced by ethanol. HepG2 cells presented numerous condensed nuclei, one of morphological changes of apoptosis, when exposed to 5% ethanol for 2 days (photographs not shown here). When the degree of cell death was assessed by MTT assay (Fig. 1B), 74% of total cells were dead due to exposure to ethanol. FSP suppressed ethanol-induced cell death in a dose-dependent manner, with a maximal

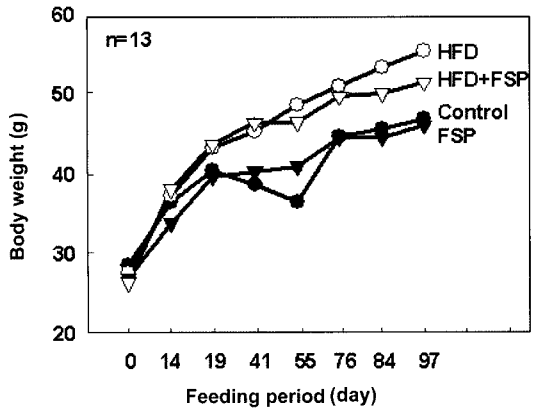


**Fig 2. Changes in body weight of mice fed various diets.** FSP, fermented *Citrus sunki* peel; HFD, high-fat diet.

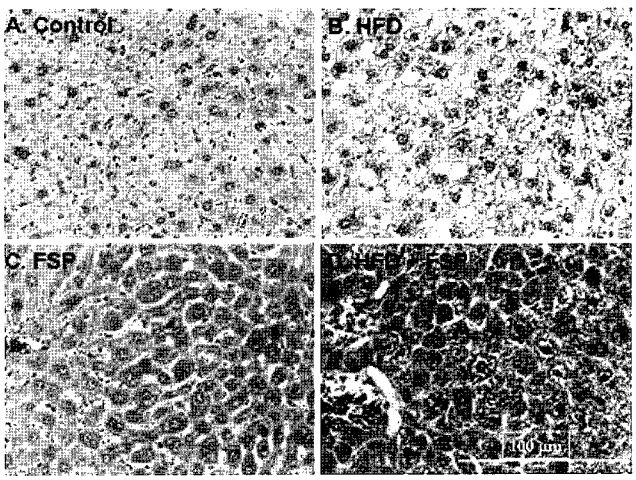


**Fig. 3. Histological changes in the liver of mice fed various diets for 97 days.** FSP, fermented *Citrus sunki* peel; HFD, high-fat diet.

protection showing a 1.36 fold increase in viability compared to the control without ethanol. This result also suggests that FSP can protect cells from apoptosis induced by serum-deprivation (11) as well as by the presence of excessive ethanol (19) in the culture medium.

**Changes in body weight and liver histology** Male ICR mice were fed with a high-fat diet for 97 days to induce obesity and fatty liver formation. Animals were divided into 4 groups (n=13 each) (control; FSP; high-fat diet, HFD; HFD+FSP). The average body weight of 52 mice at the start of the experiment was 27.43±1.35 g. Body weight was measured every week and histological changes in the liver were observed following sacrifice after 97 days on the high fat diet. The final body weight (at day 97) of each group was, 46.98±2.78 g (control), 46.12±3.14 g (FSP), 55.45±8.28 g (HFD), and 51.52±5.37 g (HFD+FSP) (Fig. 2). Although the difference between the control and FSP groups was not statistically significant, supplementation of the HFD with FSP significantly reduced the body weight gain induced by HFD alone ($p<0.05$). Anatomical observation also showed marked enlargements in various fat bodies including visceral, epididymal, and renal fat bodies (figure not shown). This result suggests that FSP serves to suppress body fat formation or to stimulate lipolysis. The exact role of FSP in fat metabolism remains to be determined. Together with the enlargement of fat bodies, severe fatty liver formation was also observed after 97 days on the HFD (Fig. 3). Surprisingly, a number of small-sized lipid droplets were also observed in the liver tissues of mice fed a control diet. Supplementation of the diet with FSP completely eliminated lipid droplets in the liver tissues of mice fed a control or high fat diet.

As shown previously, supplementation with the well-known citrus flavonoids naringin or naringenin failed to suppress body weight increase induced by high-cholesterol, whereas it slightly lowered hepatic acyl-coA:cholesterol acyltransferase (ACAT) activity which plays a major role in the regulation of cholesterol metabolism (20). However, the amounts of total cholesterol and triglyceride were not changed by naringin or naringenin in the same animal models. The product of fermented *C. unshiu* significantly suppressed body weight increase induced by a high-fat diet for 42 days in our previous study (12). Thus, it is not clear which components in citrus fruits are responsible for the suppression of body weight increase induced by a high-fat diet. Although it is suggested that some flavonoids, including hesperetin, its derivatives, and naringin, suppress HMG-CoA reductase or ACAT activity thereby serving to reduced the amount of body fat (21), novel components other than flavonoids in citrus fruits or their fermented products may play more essential roles in that process.

Overall, FSP appears to have a variety of beneficial functions including the facilitation of ethanol metabolism, and the suppression of obesity and fatty liver formation induced by a high-fat diet. FSP may therefore be a potent source of neutraceuticals beneficial for preventing ethanol-induced health problems.

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